Separation of Kentucky Bluegrass Cultivars using Peroxidase Isoenzyme Banding Patterns[1]

D. J. Wehner, J. M. Duich, and T. L. Watschke[2]

ABSTRACT

The peroxidase isoenzyme banding pattern of 15 Kentucky bluegrass (*Poa pratensis* L.) cultivars was investigated for use in identification. Isoenzymes were separated by polyacrylamide slab gel electrophoresis, stained with benzidine-H_2O_2, and the relative intensities of the bands evaluated with a densitometer. Cultivar separations were made on single seedling samples at the 1% level of confidence using band number, mobility, and intensity ratios. Overall variability of the procedure was evaluated using a horeradish peroxidase standard and the effects of seedling age and seedlot determined. Of the 15 cultivars, 11 could be separated individually, whereas the remaining 4 were grouped into two pairs. Band mobilities for the standard and the cultivars varied 1 to 3%, whereas intensity ratios varied 9% for the standard, and 5 to 20% for most cultivars. Neither seedlot, seedling age, nor leaf position on the same seedling affected the peroxidase isoenzyme banding pattern.

Additional index words: ***Poa pratensis*** **L., Electrophoresis.**

THE need for precise descriptions of plant cultivars has intensified with passage of the Plant Variety Protection Act of 1970. In the past, cultivar identification was primarily based on visual descriptions of plants or seeds (2, 9). Development of new cultivars which lack distinguishing morphological characteristics has emphasized the need for more positive identification. A chemical or biochemical test which could identify cultivars based on quantitative characteristics would be most useful. Efforts to utilize chemical or biochemical differences for cultivar characterization were reviewed by McKee (3).

The protein content of Kentucky bluegrass (*Poa pratensis* L.) has been studied for identification purposes (1, 8). Polyacrylamide gel electrophoresis was used to separate the proteins which were then stained with Commassie Blue. Several (8) or all (1) of the cultivars used failed to show reproducible banding patterns.

There have been indications that isoenzyme composition may be useful for identification purposes (3). Moberg (4) investigated the peroxidase isoenzyme banding patterns of 17 Kentucky bluegrass cultivars using starch gel electrophoresis. He made separations based on position and intensity of the stained isoenzymes on the gel. However, limitations in procedure did not allow quantification of cultivar differences. The objectives of this study were: a) to reexamine the peroxidase isoenzyme composition of Kentucky bluegrass by using improved equipment and procedures to quantify cultivar differences; and b) to assess the effect of age, intraplant variability, and seedlot on the peroxidase pattern.

MATERIALS AND METHODS

The cultivars and their seed sources used in this study are listed in Table 1. Seeds were blotter germinated in a Cleland model 500TL germinator (8 hours light at 30 C and 16 hours dark at 15 C). Seedlings were transplanted into 5 × 5 × 5 cm pot-paks filled with a 50:50 (vol/vol) mix of washed river sand and Jiffy Mix. The pots were placed in a growth chamber (12 hours light, 1,560.8 lux, at 26 C and 12 hours dark at 21 C) for 21 days to guarantee enough single seedling tissue (no roots) for extraction. Tissue was lyophilized, weighed, and stored in a desiccator at —4 C.

The peroxidase isoenzymes were extracted by grinding tissue with 0.075 *M* tris-sulfate buffer (0.2 ml buffer/mg tissue), pH 9.0, with a mortar and pestle. The slurry was centrifuged at 30.000 × *g* for 30 min at 4 C and 80μl of the supernatent was used for electrophoresis.

Acrylamide gel casting and operating procedures for the Ortec 4010 system (Ortec Inc., 100 Midland Road, Oak Ridge, TN 37830) used in this study are described in the equipment instruction manual (5). A 7.5% separating gel and a 4.5% stacking gel were used. The gel buffer was 0.375 *M* tris-sulfate and the tank buffer was 0.065 *M* tris-borate, both at pH 9.0. Electrophoresis was terminated when a bromphenol blue dye marker had migrated to the bottom of the gel. Gels were stained for peroxidase in a manner similar to that of Thorup et al. (6). However, the following modifications were made in this research. Benzidine dihydrochloride (0.25 g) was mixed with 2 ml of glacial acetic acid which was then added to 250 ml of deionized water. This solution was placed in a constant temperature water bath (25 C) and 5 ml of 20% hydrogen peroxide was added. Gels were stained for 15 min. The modified stain contained less benzidine than the stain of Thorup et al. (6) but gave similar results. No destaining was required.

Several common bands developed near the origin for all cultivars and were of no value in separation. However, two to four additional bands, depending on cultivar, were found

Table 1. Source of seed.

Cultivar	Source of Seed
Delft	1972 Northeast Regional Bluegrass Test†
Parade	1972 Northeast Regional Bluegrass Test
Galaxy	1972 Northeast Regional Bluegrass Test
Sydsport	1972 Northeast Regional Bluegrass Test
Windsor	1972 Northeast Regional Bluegrass Test
Glade	1972 Northeast Regional Bluegrass Test
Nugget	1972 Northeast Regional Bluegrass Test
Fylking	1972 Northeast Regional Bluegrass Test
Pennstar	1967 and 1968 Breeder's Seed, Pennsylvania AES, USA
Newport	Foundation Lot M12-6-66, and Certified Lot M12-6-73
Merion	1972 Northeast Regional Bluegrass Test, 1973 Breeder's Seed, and Certified Lot M65-5-M12A
K-150A	1966 Parent Plant, 1972 Second Generation grown in University Park, Pa., and 1973 Second Generation grown in Oregon
K-169	1965 Parent Plant, 1972 Second Generation grown in Oregon, and 1973 Second Generation grown in University Park, Pa.
K-170	1965 Parent Plant
K-190	1967 Parent Plant, and 1973 Second Generation grown in University Park, Pa.

† Seed supplied by agencies holding proprietary rights.

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[2] Graduate assistant, professor, and associate professor, respectively. The Pennsylvania State Univ., University Park, Pa.

to be useful for separation. The mobility of these bands was determined by the equation:

$$M = (B_d/D_d) \times (Gl_b/Gl_a)$$

where M was mobility, B_d the distance of the band from the origin, D_d the distance of the marker dye from the origin, Gl_b the gel length before staining, and Gl_a the gel length after staining.

Stained gels were scanned on a Clifford model 445 densitometer set at 450 nm with slit dimensions of 1.5 × 0.15 mm. Scans were made from the bottom (anode) to the top (origin) to facilitate zeroing of the densitometer. Horseradish peroxidase (HRP) was included in each run and scanned as a standard for each gel. Peak heights (mm) measured from densitometer tracings were used to calculate intensity ratios for all combinations of useful bands for each cultivar.

The cultivars were grouped according to band number for statistical analysis. A completely random design with unequal replication (cultivars were run 6 to 12 times) was used with a separate analysis for each mobility and ratio in the group. Coefficients of variation (C.V.) for the mobilities and intensity ratios were also calculated.

Gel Variability. Within gel variability was evaluated by running 10 subsamples from one Pennstar extraction. Gel-to-gel variation was checked by running a HRP standard in all gels used in the identification experiments. This standard reflected any difference in gels and staining procedure. Sample variation was studied by running nine individual samples from the same Pennstar seedlot on one gel. Coefficients of variation for the mobilities and intensity ratios were calculated.

Seedlot Variability. Samples from two Merion, Pennstar, and Newport and three K-150A and K-169 seedlots were run in

Table 2. Band mobilities and intensity ratios for the six Kentucky bluegrass cultivars in the four band grouping.

Cultivar	Mobility of band†				Intensity ratio					
	1	2	3	4	2/1†	3/1	4/1	2/3	2/4	3/4
Delft	0.61 a*	0.56 b	0.50 b	0.42 a	0.53 d	0.59 c	0.70 b	0.90 b	0.76 c	0.85 d
Nugget	0.62 a	0.51 c	0.48 c	0.43 a	1.58 a	1.37 a	1.40 a	1.16 b	1.14 bc	0.99 cd
Glade	0.62 a	0.57 b	0.52 a	0.43 a	0.82 b	0.98 b	0.75 b	0.85 b	1.11 bc	1.32 a
K-150A	0.62 a	0.57 ab	0.51 ab	0.43 a	0.66 cd	0.23 d	0.51 c	3.10 a	1.37 b	0.51 e
Windsor	0.62 a	0.57 ab	0.52 a	0.43 a	0.84 b	0.28 d	0.25 d	3.18 a	3.46 a	1.17 bc
K-169	0.63 a	0.58 a	0.52 a	0.43 a	0.71 bc	0.21 d	0.51 c	4.00 a	1.42 b	0.43 e

* Within columns, values having a letter in common are not significantly different at the 1% level by the Duncan's modified L.S.D. test.
† Bands were numbered separately for each cultivar from anode to origin.

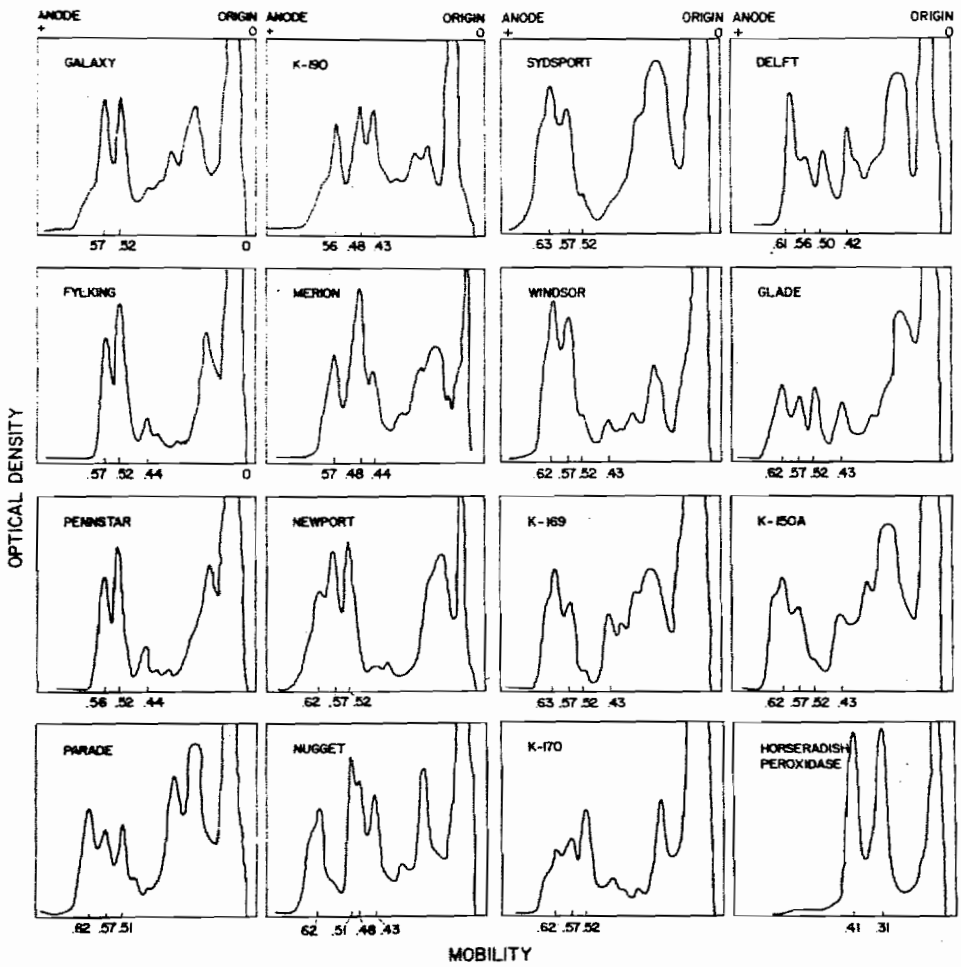

Fig. 1. Densitometer tracings of the peroxidase isoenzyme banding patterns for the 15 Kentucky bluegrass cultivars. Mobilities are indicated for bands used in separation.

adjacent wells of a gel to test for variability in the peroxidase isoenzyme patterns due to seedlot. Visual inspections of the gel and densitometer tracings were made.

Intraplant Variability. Individual leaves were separated from several similar sized Merion seedlings. Leaves from the oldest to the youngest were combined according to age and were run on the same gel.

Effect of Off-type. Four off-types were selected in a Pennstar foundation seed field in Oregon. Based on appearance, they were believed to have been the result of an unreduced egg of Pennstar being fertilized by a reduced pollen gamete of Pennstar in an isolated breeder's nursery. Each was found to be highly apomictic, and showed only slight phenotypic differences when grown as field space plants. Samples of Pennstar and these off-types were grown, prepared, and run as previously described.

Age Variation. The effect of seedling age on the peroxidase isoenzyme pattern was studied using Newport and Merion. Seeds were placed in the germinator each day for 3 weeks and 9 days after start of germination were placed in the growth chamber. At the end of 1 month, seedlings ranging in age from just germinated to 21 days in the growth chamber were harvested and lyophilized. Seedling samples of the same age were combined to provide enough tissue for extraction. A large sample (4 mg) was extracted in 0.2 ml buffer to allow for bulk sampling of older tissue also. Supernatent (40 μl) was applied to the gel and current was passed until the fourth band of a myoglobin marker had migrated 2 cm.

Table 3. Band mobilities and intensity ratios for the eight Kentucky bluegrass cultivars in the three band grouping and Galaxy.

	Mobility of band†			Intensity ratio		
Cultivar	1	2	3	2/1†	3/1	2/3
Sydsport	0.63 a*	0.57 a	0.52 a	0.89 de	0.23 e	4.02 a
Parade	0.62 a	0.57 a	0.51 a	0.81 e	0.95 c	0.86 d
K-170	0.62 a	0.57 a	0.52 a	1.38 bc	2.15 a	0.65 d
Newport	0.62 a	0.57 a	0.51 a	1.41 b	1.48 b	0.95 d
Fylking	0.57 b	0.52 b	0.44 b	1.33 bc	0.43 de	3.14 b
Merion	0.56 b	0.48 c	0.44 b	1.71 a	0.91 c	1.90 c
Pennstar	0.56 b	0.52 b	0.43 b	1.25 c	0.47 d	2.79 b
K-190	0.56 b	0.48 c	0.43 b	1.05 d	1.04 c	1.01 d
Galaxy‡	0.58	0.52		0.99		

* Within columns, values having a letter in common are not significantly different at the 1% level by the Duncan's modified L.S.D. test. † Bands were numbered separately for each cultivar from anode to origin. ‡ Galaxy had only two characteristic bands and was not included in the AOV.

RESULTS AND DISCUSSION

Separation of 15 Kentucky Bluegrass Cultivars

Peroxidase isoenzyme banding patterns differed considerably among the 15 cultivars tested, although several common bands were found for all cultivars. Initial separation was made according to the number of bands useful for identification. 'Galaxy' had two characteristic bands; 'Fylking,' 'Pennstar,' 'Parade,' K-190, 'Merion,' 'Newport,' K-170, and 'Sydsport,' three; 'Windsor,' K-169, 'Nugget,' 'Delft,' 'Glade,' and K-150A, four (Fig. 1). Some cultivars showed similarities in the nature and number of peroxidase isoenzymes present. However, separation was also made on the basis of relative differences in the activity of the isoenzymes for each cultivar. For example, Windsor and K-150A had four bands with the same mobilities, but by comparing the intensity ratio of bands 2 and 4 (2/4), they could be separated. Tables 2 and 3 show the mobilities and intensity ratios for all cultivars. Newport and K-170 had three bands in the same position, however, their intensity ratios were different. Three cultivars (Merion, Nugget, and Delft) had distinct banding patterns and could be consistently recognized without densitometer tracings.

By using band number, mobility, and intensity ratio differences, the uniqueness of individual peroxidase

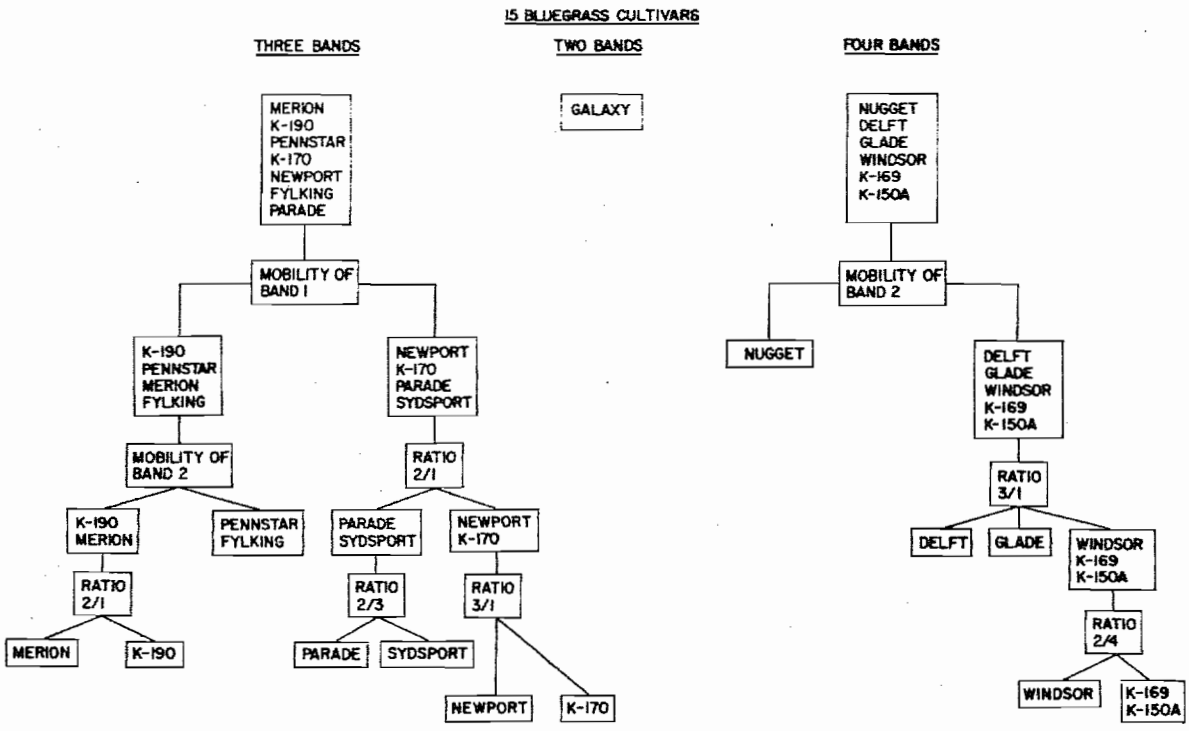

Fig. 2. Flowchart for separating the 15 Kentucky bluegrass cultivars used.

Table 4. $(\bar{x})$ and coefficients of variation (C.V.) for the mobilities and peak height ratios of Pennstar Kentucky bluegrass and horseradish peroxidase.

	Mobilities					
	Band 1		Band 2		Band 3	
	$\bar{x}$	C.V. (%)	$\bar{x}$	C.V. (%)	$\bar{x}$	C.V. (%)
Pennstar (9 individual samples on one gel)	0.55	1.42	0.50	1.56	0.42	1.65
Pennstar (10 subsamples on one gel)	0.58	1.62	0.53	1.72	0.46	1.45
Horseradish peroxidase (11 subsamples on 11 gels)	0.32	2.60	0.41	2.27		
	Intensity ratio					
	2/1		3/1		2/3	
	$\bar{x}$	C.V. (%)	$\bar{x}$	C.V. (%)	$\bar{x}$	C.V. (%)
Pennstar (9 individual samples on one gel)	1.36	7.10	0.41	25.70	3.57	27.19
Pennstar (10 subsamples on one gel)	1.50	3.80	0.55	11.70	2.77	10.13
Horseradish peroxidase (11 subsamples on 11 gels)	1.00	9.10				

Table 5. Coefficients of variation for the intensity ratios of the eight Kentucky bluegrass cultivars in the three band grouping and Galaxy.

	C. V.		
	Intensity ratio		
Cultivar	2/1†	3/1	2/3
	%		
Sydsport	7.50	18.18	13.51
Parade	10.58	14.27	5.30
K-170	22.58	25.87	7.66
Newport	5.83	6.72	2.94
Fylking	7.72	9.15	12.12
Merion	8.89	9.86	7.55
Pennstar	4.68	21.07	26.59
K-190	13.00	6.88	11.37
Galaxy	2.59		

† Bands were numbered separately for each cultivar from anode to origin.

Table 6. Coefficients of variation for the intensity ratios for the six Kentucky bluegrass cultivars in the four band grouping.

	C. V.					
	Intensity ratio					
Cultivar	2/1†	3/1	4/1	2/3	2/4	3/4
	%					
Delft	9.38	6.09	7.93	12.44	14.30	6.86
Nugget	10.78	10.28	9.69	13.80	14.98	12.71
Glade	13.53	10.74	17.33	15.63	14.65	14.82
K-150A	24.05	29.56	31.70	42.37	29.51	48.02
Windsor	12.56	30.51	17.48	26.32	21.60	38.93
K-169	27.32	47.71	25.70	55.12	16.37	42.01

† Bands were numbered separately for each cultivar from anode to origin.

patterns was ascertained and a flow chart (Fig. 2) was constructed to show the cultivar separation. Eleven of the 15 cultivars used in this experiment were separated individually. The three-band cultivars Fylking and Pennstar could not be separated, nor could the four-band K-150A and K-169. Fylking and Pennstar are known to be phenotypically similar, but the latter two are quite distinct. Although these two pairs cannot be separated by peroxidase isoenzyme pattern, their obvious genetic divergence may be manifest in some other isoenzyme system. Efforts to further separate these grasses using esterase isoenzyme patterns were unsuccessful due to limited banding.

Variability

Sample and technique variation were a major concern in interpreting data. Coefficients of variation and means for the mobilities and intensity ratios measured in the sample and technique variation studies are shown in Table 4. Mobility showed little variation within and between gels and was the most reliable indicator of cultivar differences. The C.V.'s were less than 3.5% for all cultivar mobilities.

The amount of variation in the intensity ratios is important since several cultivars had bands in the same position but differed in their relative intensities. The C.V. for the first intensity ratio of Pennstar (Table 4, Sample and Technique Study) was 7.1%; however, the C.V.'s for the second and third ratios were quite large (26 and 27%). The second and third ratios also had higher C.V.'s than the first when subsamples were used. Apparently, band three of Pennstar was sensitive to within gel variation. Variability of the intensity ratio of the HRP standard (9.1%) was similar to that for the subsample run of Pennstar. Therefore, it appeared that a minimum of 10% variation in the intensity ratios could be expected. Variation above 10% was attributed to the variability within the cultivar. The variation in the intensity ratios shown by all cultivars are listed in Tables 5 and 6. Although several cultivars (K-170, K-150A, K-169, and Windsor) exhibited a large variation in their intensity ratios, the variation was not large enough to prevent separation by statistical procedures. Other than the pairings of K-150A and K-169, and Pennstar with Fylking, only K-170 and Newport showed the possibility of overlap for the intensity ratio used to separate them. If both showed the maximum variation for the intensity ratio 3/1, the values for this ratio would coincide. However, these cultivars were separated by Duncan's modified L.S.D. test (7) at the 1% ($K = 500$) level as indicated in Table 3.

There were no differences due to seedlot in the peroxidase patterns of Merion, Newport, Pennstar, K-150A and K-169. The mobilities and intensity ratios showed only the normal variation. One seedlot of K-150A showed more within lot variability than the other two, but the basic pattern was the same for all three lots. The results indicated that separation of these cultivars was not affected by different seedlots.

Intraplant Variability

The peroxidase isoenzyme bands for the individual leaves of Merion showed the same mobilities as those of the whole plant sample. Individual leaves did show activity differences. The second leaf had wider and darker bands than the others. The intensity ratios for individual leaves did not differ from those found for Merion in other runs. Therefore, any foliar portion can be used for analysis.

Age

An important consideration in any cultivar identification procedure is the time needed to grow the plant and perform the test. If the same time allotted for a

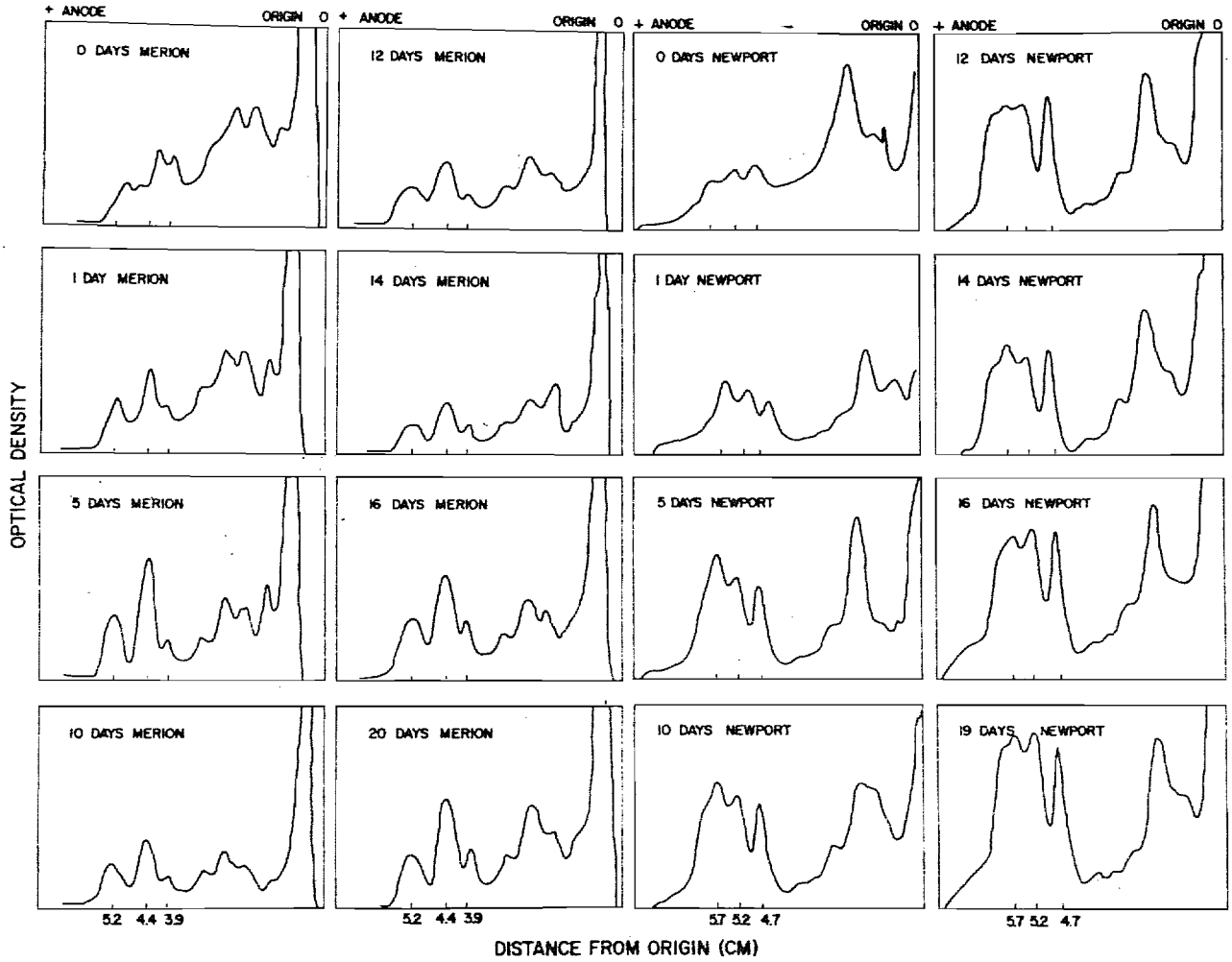

Fig. 3. Densitometer tracings of the peroxidase patterns of Merion and Newport Kentucky bluegrass at several seedling ages. Time in growth chamber is indicated. Seedlings were placed in growth chamber 9 days after start of germination.

standard seed germination test was available for varietal purity testing, 28 days could be used to grow and analyze a bluegrass sample. Minimum sample size for the isoenzyme procedure is 1.5 mg of lyophilized tissue. A seedling of the necessary size can be grown in 22 to 28 days from seed or 12 to 18 days after it is in the growth chamber. The position and relative intensities of the isoenzymes must remain constant during this time so that seedlings of various ages can be identified correctly.

The characteristic peroxidase isoenzyme bands did not noticeably change with age for Merion and Newport seedlings (Fig. 3). There was however, a slight change in the banding pattern as the seedlings were taken from the germinator to the growth chamber when characteristic bands became darker. The mobilities of the bands were not calculated because the bromphenol blue dye marker came off the gel before the runs were finished. Current was passed for a longer period of time in the age study so that the bands would be spread out more on the gel to reveal any slight differences. The gels showed no difference due to age in the distance of the bands from the origin. For the 12 to 18 day period when seedlings were the necessary size for sampling, the three intensity ratios for Merion varied 7.3, 9.0, and 9.9% while Newport's varied 6.1, 4.9, and 3.8%. This variation is approximately equal to that shown by Merion and Newport samples of the same age (Table 5.). Therefore, seedling age had no apparent effect on cultivar separation.

Pennstar Off-types

It was of interest to know if the peroxidase patterns of progeny off-types were different. Pennstar and its four off-types exhibited the same banding pattern. The mobilities were the same, and while values for the intensity ratios were different, they were in the range of those shown by Pennstar in previous runs. It was concluded that these off-types could not be distinguished from Pennstar by their peroxidase isoenzyme pattern. Since morphological differences were used to select these off-types from Pennstar, it may be possible to use some other isoenzyme to separate them.

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